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CONVERSION OF 12-HYDROXYOCTADECANOIC ACID TO 12,15-, 12,16- AND 12,17-DIHYDROXYOCTADECANOIC ACIDS WITH Bacillus sp. U88

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SUMMARY

A new microbial isolate, *Bacillus sp.* U88, transformed 12-hydroxyoctadecanoic acid to 12,15-, 12,16- and 12,17-dihydroxyoctadecanoic acids when grown aerobically in 1% yeast extract medium at 30°C and shaken at 250 rpm for 5 days. These three dihydroxyfatty acids were derivatized with Trimethylsilyl ether and identified by GC-MS analysis. The yield of 12,15-, 12,16- and 12,17-dihydroxyoctadecanoic acids after 5 day incubation were 2.8%, 7.1% and 1.1%, respectively.

INTRODUCTION

Mono- and polyhydroxyfatty acid derivatives have potential uses as lubricants, detergents and other commercial products (Dahlke et al., 1995). *B. megaterium* transforms saturated and unsaturated fatty acids (C14 to C20) to their corresponding monohydroxy fatty acids at ω-1, ω-2 and ω-3 (Miura and Fulco,1974), *B. pumilus* transforms 9(Z)-octadecenoic acid (oleic acid) to 15-, 16-, and 17-hydroxy-9(Z)-octadecenoic acids (Miura and Fulco,1974; Lanser *et al.*,1992), and microorganisms transforming oleic acid to 12-hydroxy-9(Z)-octadecenoic acid (ricinoleic acid) (Soda,1987; Soda and Kido, 1989) also have been reported. Regioselective oxidation of dodecanoic acid (lauric acid) to 9-, 10- and 11-hydroxylauric acid by cytochrome P-450 hydroxylase occurs in most plants, particularly in wheat (Durst *et al.*, 1992).

During our continuing efforts to isolate new microorganisms from soil, water, or compost manure, and to carry out

biotransformation of 12-hydroxyoctadecanoic acid (12-HOA) by isolates (Huang *et al*,1995), we discovered a new active strain of *Bacillus* U88. *Bacillus sp.* U88 produced reasonable amounts of three dihydroxyoctadecanoic acid isomers when grown aerobically in 1% yeast extract (1% YE) medium at 30°C, 250 rpm, in the presence of 0.2% (W/V) of 12-HOA. We report in this paper: (a) Isolation of three dihydroxyfatty acid isomers from conversion products by thin-layer chromatography (TLC) or high pressure liquid chromatography (HPLC); (b) Identification of these three isomers by GC-MS of their trimethyl silyl derivatives; and (c) The bioconversion yield of dihydroxyfatty acids after 2 and 5 days incubation with 12-HOA.

MATERIALS AND METHODS

Materials. All chemicals and solvents were ACS grade obtained from commercial sources. The purity of R-(+)-12-hydroxyoctadecanoic acid (12-HOA), erythro-9,10-dihydroxyoctadecanoic acid (9,10-DHOA) and hexadecanoic acid (palmitic acid) were greater than 98% by GC analysis. Yeast extract and dehydrated skim milk were from Difco Laboratories(Detroit, MI).

Microorganisms and microbial conversion studies. Strain U88 was isolated from the intestinal tract of a fish (Kyaw, 1991). A single colony was cultured in 5 ml of tryptone medium (containing 0.5% tryptone, 0.2% KH_2PO_4, 0.4% Na_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.01% $CaCl_2.H_2O$ and 0.001% $FeSO_4.7H_2O$, pH 7.2.) at 30°C, 200 rpm for 18 hours. The following day, 2 ml of culture was added to 100 ml of 1% YE medium to make a 1 to 50 dilution. Ten ml of dilution culture per 125 Erlenmeyer flask was made and 12-HOA was added to a final concentration of 0.2% to carry out the bioconversion reaction for up to 5 days. Flasks containing only dilution culture served as control.

Samples (0.5 ml) were removed on day 2 and day 5 and were acidified to pH 3 with 3 N HCl. Two hundred ug of palmitic acid was added as internal standard, and then the sample was extracted twice with an equal volume of diethyl ether. The combined ether layers were washed once with an equal volume of deionized water prior to solvent removal under nitrogen.

For large scale culture, a 1 to 50 dilution culture (from an overnight culture) was grown aerobically in a one liter flask containing 250 ml of 1% YE medium (total 500 ml) and 12-HOA was added (final concentration 0.2%, W/V) and incubated for 5 days. Samples of 0.5 ml were taken on day 2 and day 5 for GC analysis. Samples were extracted with ether and worked up in the usual manner.

Medium preparation. The 1% YE medium contained 1% yeast extract, 0.2% KH_2PO_4, 0.4% Na_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.01% $CaCl_2.H_2O$ and 0.001% $FeSO_4.7H_2O$, pH 7.2. Autoclaved medium was freshly prepared for each use.

Thin-layer chromatography. Crude extracts were subjected to partial purification before applying to TLC plates. Crude extracts were dissolved in minimum amounts of methanol in a flask, and diethyl ether was added to a final conc. of 98% (V/V). The flask was kept at -20°C for 1 hr. The precipitate was collected and dried under N_2. Dried samples were redissolved in methanol and

reprecipitated once as above. Partially purified samples in methanol were spotted on 20 X 5 cm Silica gel 60 plate (0.25 mm thickness; EM Science, Cherry Hill, NJ) as a band 2 cm from the bottom of the plate and developed with CH_2CL_2/MeOH/CF_3COOH (97/2/1) system. The area containing the dihydroxyfatty acids was removed from the glass plates, extracted with methanol and dried with N_2.

Preparative HPLC. Preparative chromatography was conducted isocratically on HPLC (Millipore Corp.) with CH_2CL_2/MeOH/CF_3COOH (97/2/1) system at 3 ml per minute. A 58 mg sample in methanol was injected onto a Dynamax-60A silica column (25cm x 41.4mm I.D., Rainin Instrument Co). Effluent was monitored serially, by ultraviolet absorbance (245 nm) and refractive index. A total of 9 fractions were collected. The solvent was removed under nitrogen. Samples of each fraction were methylated with diazomethane and analyzed by gas chromatography. The 9th fraction was the 100% methanol wash. Most of the dihydroxyfatty acid isomers appeared in this fraction with 95% purity by GC. Compounds in this fraction were further purified by precipitating them in a methanol/ether mixture (2/98=V/V) as mentioned above and subjected to IR, GC-MS (methyl- and TMS-derivatives) and PCI-MS analyses.

Fourier Transform Infrared (FTIR) Spectroscopy. Infrared spectra of the free acids (thin film, KBr plate) were obtained with a Mattson Galaxy 6020 IR spectrometer (Mattson Instruments, Inc., Madison, WI).

Mass Spectra (GC-MS). Crude extracts, partially purified samples from crude extract and highly purified compounds were either methylated or methylated followed by trimethyl silylation prior to GC and GC-MS analyses. Esters were separated on a SPB-1 column (15m x 0.32 mm I.D. and 0.25um thickness, Supelco, Co) either isothermally at 220°C or temperature programmed as previous;y reported (Huang et al.,1995). Peak areas were determined with a Hewlett-Packard 3396A electronic integrator. Methyl 9,10-dihydroxyoctadecanoate and methyl palmitate were used as standards for quantitative analyses.

Electron impact mass spectra of methyl esters and trimethylsilyl (TMS) derivatives were obtained on a Hewlett-Packard 5970 gas chromatograph equipped with DB-5MS capillary column coupled to a Hewlett-Packard mass selective detector. Mass spectrometry by Positive chemical ionization was carried out on dihydroxyoctadecanoic acid isomers by *The Nebraska Center for Mass Spectrometry*, Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE. Reagent gas was isobutane. Exact mass measurements were made using a narrow voltage scanning and ions of perfluorokerosene as standards mass.

RESULTS AND DISCUSSION

Identification of microorganisms. Strain U88 was obtained from the intestinal tract of a fish (largemouth bass, *Micropterus salmoidef*). The isolate is a gram-positive, spore-forming rod. The bacterium was positive for catalase, oxidase, nitrate reduction, and phenylalanine deaminase. Negative results were found for indole, methylene red, Voges-Proskauer, citrate utilization, and starch and gelatin hydrolysis. Based on these

reactions, the isolate was assigned to the genus Bacillus, but was unidentifiable as to species (Gibson and Gordon,1974).

Identification of conversion products. *Bacillus sp.* U88 transformed 12-HOA to several products whose methyl esters had a relatively longer GC retention time when compared to methyl 12-HOA. One of these products was purified by HPLC. It turned out that the purified product was a mixture of three dihydroxyfatty acid isomers (see below for details) which could not be separated by silica column.

IR analysis of the purified compound indicated the presence of hydroxy group (3400 cm-1) and carbonyl group (1712 cm-1). Methyl esters of purified product were not completely resolved on the SPB-1 capillary column (Fig. 1A). However, their TMS derivatives were separated into three distinctive peaks (Fig. 1B).

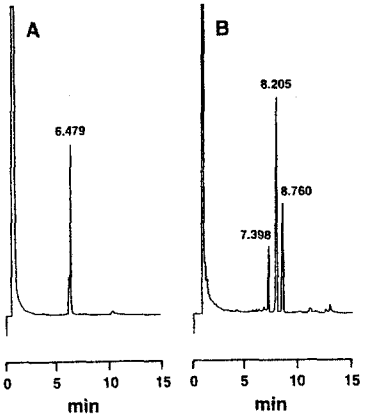

Fig.1. GC chromatograms of purified dihydroxyoctadecanoic acids. Dihydroxyoctadecanoic acids were purified from 5 day conversion poroducts as mentioned in *Materials and methods*. (A). methyl esters. (B). methyl TMS derivatives. Samples were separated on SPB-1 column isothermally at 210°C.

Electron impact mass spectra of TMS-derivatives indicated that the purified product is a mixture of three dihydoxyfatty acids: 12,15-, 12,16- and 12,17-dihydoxyoctadecanoic acids. The locations of the hydroxy groups were determined by the presence of prominent ions from α-cleavage of the sigma bonds on the left and right of the TMS groups (Kleiman and Spencer,1973). For the methyl silylated 12,15-derivative (Fig. 2A), major fragment ions were M/Z 341(36%), 301(54%, 431-90), 185(100%; 275-90) and 145(63%). Loss of 90 mass units corresponds to loss of TMSOH group. For the methyl silylated 12,16-derivative (Fig. 2B), similar fragment ions were m/z 301(65%), 275(38%), 185(18%) and 131(57%). For methyl silylated 12,17-derivative (Fig. 2C), these fragments ions were M/Z 301(100%), 275(59%), 185(18%) and 117(63%). The mass spectra of a mixture of three monohydroxyfatty acid isomers: 15-, 16- and 17-hydroxy-9(Z)-octadecenoic acid has been reported (Lanser et al., 1992). In that paper, the characteristic fragment ions M/Z 145, 131 and 117 resulted from the cleavage of the sigma bond between C14-C15, C15-C16 and C16-C17 which are identical to the fragment ions of M/Z 145, 131 and 117 seen in Fig. 2A, 2B and 2C.

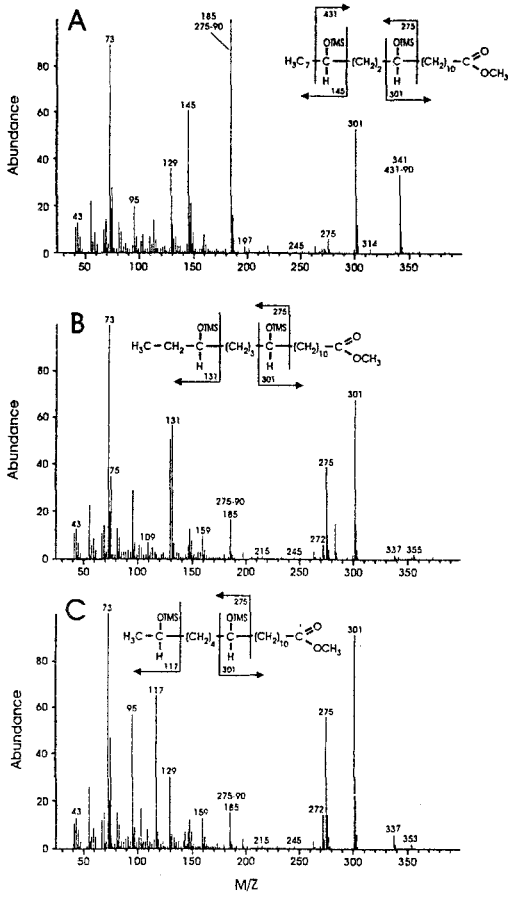

Fig. 2. Mass spectra of TMS derivatives of (A) methyl 12, 15-, (B) methyl 12,16-, and (C) methyl 12, 17-dihydroxyoctadecanoate with fragmentation pattern corresponding to specific ions.

The purified product containing three dihydroxyfatty acid isomers was subjected to PCI-MS analysis. In the CI mass spectrum, the M/Z 317(100%, $(M+H)^+$) ion corresponds to the protonation of parent molecule. The M/Z 299(100%, 317-18), 281(50%, 299-18), 263(17%, 281-18) ions correspond to loss of one, two and three H_2O from M/Z 317. In addition, the fragment ion at M/Z 215(9%) resulted from cleavage of the sigma bond at C_{12}-C_{13}. Loss of one H_2O resulted in M/Z of 197. Although the purified product was a mixture of three isomers, they do not interfere to each other by PCI-MS analysis. All isomers have a chance to be protonated (M/Z 317 $(M+H)^+$), and then subsequently to loss of one, two and three H_2O (M/Z 299, 281 and 263, respectively).

Production of Dihydroxyfatty acids. A dilution culture (1 to 50 dilution from an overnight culture) containing 0.2% of 12-HOA was grown aerobically at 30°C, 250 rpm for 5 days. Samples (0.5 ml) were taken on day 2 and day 5 for GC analyses. The yield of a mixture of three dihydroxyfatty acid isomers on day 2 and day 5 were 10% and 11%, respectively. The yield was underestimated because when authentic erythro-9,10-dihydroxyoctadecanoic acid was

used to carry out the extraction, we found that the recovery was 41%. The distribution of these three isomers after a 5 day incubation were: 12,15-dihydroxyoctadecanoic acid (25%), 12,16-dihydroxyoctadecanoic acid (65%) and 12,17-dihydroxyoctadecanoic acid (10%). A cell free system of *B. megaterium* preferentially hydroxylates palmitic acid at ω-2 (50%) (Miura and Fulco, 1974), and the wheat enzyme, In Chain-Lauric Acid Hydroxylase (IC-LAH), preferentially hydroxylates lauric acid at ω-1 (72%), whereas the Jerusalem artichoke IC-LAH preferentially hydroxylates lauric acid at ω-3 (62%) (Durst *et al*,1992).

Hydroxylation of 12-HOA at ω-1, ω-2 and ω-3 positions to yield dihydroxyoctadecanoic acids provide an alternative way (by hydroxylase) to add additional functional groups to fatty acids and may have industrial application as additive or surfactant. Although the yield of dihydroxyfatty acids was low, further screening of microbial isolates may identify high yielding strains for production of dihydroxyfatty acids.

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